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Report

# Memory in *Caenorhabditis elegans* Is Mediated by NMDA-Type Ionotropic Glutamate Receptors

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## Summary

**Learning and memory are essential processes of both vertebrate and invertebrate nervous systems that allow animals to survive and reproduce. The neurotransmitter glutamate signals via ionotropic glutamate receptors (iGluRs) that have been linked to learning and memory formation [1, 2]; however, the signaling pathways that contribute to these behaviors are still not well understood. We therefore undertook a genetic and electrophysiological analysis of learning and memory in the nematode *Caenorhabditis elegans*. Here, we show that two genes, *nmr-1* and *nmr-2*, are predicted to encode the subunits of an NMDA-type (NMDAR) iGluR that is necessary for memory retention in *C. elegans*. We cloned *nmr-2*, generated a deletion mutation in the gene, and showed that like *nmr-1* [3], *nmr-2* is required for in vivo NMDA-gated currents. Using an associative-learning paradigm that pairs starvation with the attractant NaCl [4], we also showed that the memory of a learned avoidance response is dependent on NMR-1 and NMR-2 and that expression of NMDARs in a single pair of interneurons is sufficient for normal memory. Our results provide new insights into the molecular and cellular mechanisms underlying the memory of a learned event.**

## Results and Discussion

### Associative Learning in *C. elegans*

A number of learning paradigms have been developed in *C. elegans* [5–10], including salt chemotaxis learning, in which wild-type worms learn to avoid normally attractive NaCl if it is first paired with starvation [4] (Figure 1). Thus, when tested in a chemotaxis assay, the chemotaxis index (CI) (see Experimental Procedures) of conditioned worms (starved in the presence of NaCl) 10 min after conditioning was approximately −0.5 compared to 0.6 observed for mock-conditioned worms (starved in the absence of NaCl). This learned avoidance behavior weakened with time, with most of the worms reaching the source of NaCl 2 hr after conditioning (CI ~0.75) (Figure 1B). Interestingly, naive worms initially showed a greater avoidance of NaCl (CI ~0.2 at 10 min) compared to mock-conditioned worms ($p < 0.01$), suggesting that starvation in the absence of salt enhances the attraction to NaCl.

Several gene products have been implicated in the learning of salt avoidance, including HEN-1, a protein with an LDL motif that is expressed in the bilateral pair of ASE salt-sensing neurons (ASER and ASEL) [11]; CASY-1, the ortholog of calsyntenins that is specifically required in ASER [12]; and proteins involved in the insulin-like signaling pathway [13] and the Go (GOA-1) and Gq (EGL-30) pathway [14]. However, no genes have been described that contribute to the memory of the learned event.

AMPA receptors (AMPARs) and NMDARs have been implicated in learning and memory in many organisms [1, 2]. In vertebrates, neural activity influences the cycling of AMPARs in and out of synapses. This dynamic behavior is thought to modify synaptic strength and may underlie cellular mechanisms of learning and memory, such as long-term potentiation (LTP) and long-term depression (LTD) [15, 16]. Modification of AMPAR trafficking is also thought to regulate synaptic plasticity and thus learning and memory in *C. elegans* [17] and *Aplysia* [18]. In addition, NMDARs have been implicated in associative learning and memory in *Drosophila* [19, 20] and *Apis mellifera* (honeybee) [21], and disrupting NMDAR function prevents LTP and leads to changes in learning and memory in mice [22–24] and *Aplysia* [25, 26]. However, our ability to link iGluRs and memory formation to specific cells and neural circuits that control behavior is limited by the tremendous complexity of most nervous systems and the relative difficulty of achieving specific genetic perturbations. To overcome these difficulties, we undertook a genetic analysis of associative learning and memory in *C. elegans*.

### *nmr-2* Encodes a Protein with Greatest Sequence Identity to Vertebrate NR2 Subunits

To determine whether glutamatergic signaling is required for salt chemotaxis learning in *C. elegans*, we first tested the role of the GLR-1 [27, 28] and GLR-2 [29] AMPAR subunits and the NMR-1 NMDAR subunit [3]. In addition, we cloned and characterized a second gene, *nmr-2*, encoding a putative NMDAR subunit. The full-length *nmr-2* cDNA is predicted to encode a 990 amino acid protein and includes an additional 513 bp compared to that predicted by GENEFINDER analysis [30]. NMR-2 is predicted to have a membrane topology similar to that of other iGluR subunits and shares the greatest sequence identity with vertebrate NR2 subunits (Figures 2A and 2B). To study the contribution of NMR-2 to neuronal function, we generated a deletion mutation in *nmr-2* with standard techniques, first screening for insertion of the Tc1 transposon in the *nmr-2* locus and then identifying a rare imprecise excision event (Figure 2C). The *nmr-2(ak7)* deletion removes approximately 2.5 kb of genomic sequence, including that which is predicted to encode transmembrane domains II and III and the S2 domain that forms

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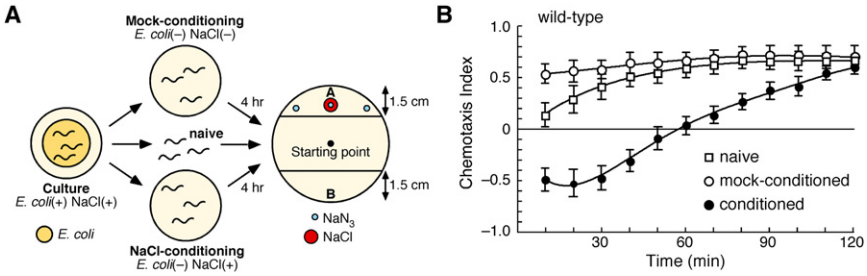


Figure 1. Salt Chemotaxis Learning in Wild-Type Worms

(A) Schematic of the salt-chemotaxis-learning assay. The starting point of naive, mock-conditioned, and conditioned worms at the beginning of the chemotaxis assay is indicated. Sodium azide ($NaN_3$) was used to paralyze animals once they reached the source of NaCl.

(B) Salt-chemotaxis-learning behavior in naive (n = 5), mock-conditioned (n = 5), and conditioned (n = 6) wild-type worms. Error bars represent SEM.

part of the ligand-binding pocket (Figure 2C). Similar to *nmr-1(ak4)* mutants [3], *nmr-2(ak7)* mutants were viable and showed no gross defects in locomotion (data not shown).

### *nmr-1* and *nmr-2* Mutants Are Defective in Salt Chemotaxis Learning

To test the role of both non-NMDA and NMDA iGluRs in learning and memory, we characterized salt chemotaxis learning in *glr-1(n2461)* [27], *glr-2(ak10)* [29], *nmr-1(ak4)* [3], and *nmr-2(ak7)* mutants. All single mutants showed normal chemotaxis to NaCl in mock-conditioned assays and avoided NaCl just after conditioning (Figures 3A–3D). Interestingly, the *nmr-1* and *nmr-2* mutants were unable to fully retain the memory of the learned behavior and recovered from the avoidance state (CI = 0 at 30 min) more rapidly than either wild-type animals (CI = 0 at 60 min) (Figure 1B) or AMPAR mutants (CI = 0 at 50 min). We also examined memory retention in double mutants. Worms with either the *glr-1(n2461)* or *glr-2(ak10)* mutation in combination with either *nmr-1(ak4)* or *nmr-2(ak7)* were not different from either the *nmr-1* or *nmr-2* single mutants (data not shown). Similarly, the *nmr-1(ak4); nmr-2(ak7)* double mutant was not significantly different from either single mutant (Figure 3E). These data suggest that NMDARs, but not AMPA-type non-NMDARs, are required for the memory component of salt chemotaxis learning and that NMR-1 and NMR-2 may combine to form a functional heteromeric NMDAR.

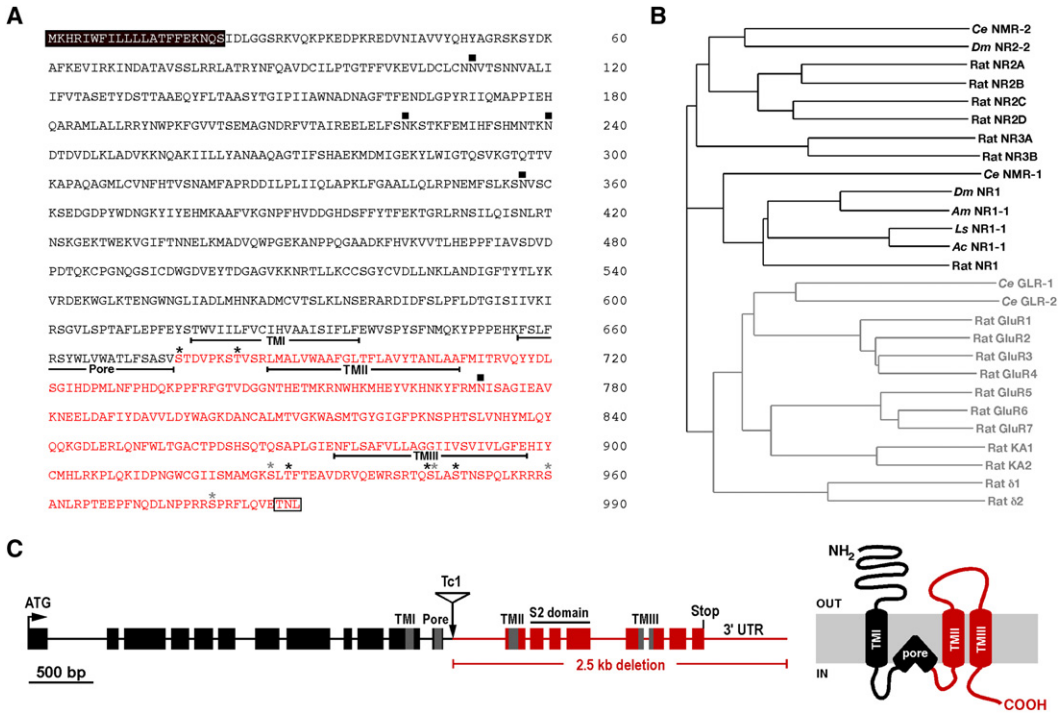


Figure 2. *nmr-2* Encodes a 990 Amino Acid Protein with Greatest Sequence Identity to Vertebrate NR2 Subunits

(A) Predicted protein sequence encoded by the *nmr-2* gene. Indicated are the putative transmembrane domains (underlined), N-linked glycosylation sites (marked by filled squares), PKA (marked gray asterisks) and PKC (marked by black asterisks) phosphorylation sites, the putative signal sequence (black box), the region deleted by the *ak7* mutation (red text), and the putative PDZ-domain-binding motif (white box).

(B) Phylogenetic tree of *C. elegans* (*Ce*), *Rattus norvegicus* (Rat), *Drosophila melanogaster* (*Dm*), *Apis mellifera* (*Am*), *Aplysia californica* (*Ac*), and *Lymnaea stagnalis* (*Ls*) iGluRs. NMDARs are denoted in black text, and non-NMDARs are denoted in gray text.

(C) Genomic organization of the *nmr-2* locus with exons and introns represented as boxes and lines, respectively (left). The site of the Tc1 insert is indicated, and the region deleted by its imprecise excision is shown in red. The approximate location of the sequence encoding the pore region and TMI–TMIII are highlighted in gray, and the S2-domain-coding sequence is shown (black line). The predicted membrane topology of NMR-2 with the region deleted by the *ak7* mutation shown in red (right).

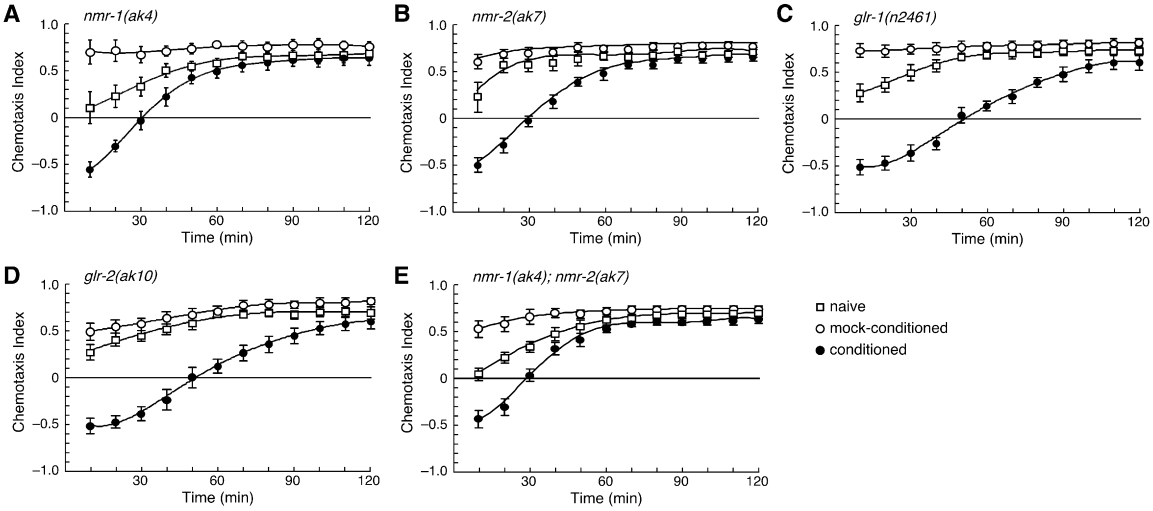


Figure 3. Retention of Avoidance Memory Is Impaired in *nmr-1* and *nmr-2* Mutants

(A–E) Chemotaxis learning in *nmr-1(ak4)* (n = 6) (A), *nmr-2(ak7)* (n = 6) (B), *glr-1(n2461)* (n = 4) (C), *glr-2(ak10)* (n = 5) (D), and *nmr-1(ak4); nmr-2(ak7)* (n = 4) (E). *nmr-1* and *nmr-2* single mutants and the *nmr-1; nmr-2* double mutant were statistically different from the wild-type at 40 min ($p < 0.001$). Error bars represent SEM.

### *nmr-1* and *nmr-2* Mutants Can Sense Food and Starvation

To ensure that the memory defects observed in *nmr-1* and *nmr-2* mutants were not due to an inability to sense starvation, we tested the behavior of both well-fed and starved mutants in the basal slowing response and the enhanced slowing response. Sawin et al. [31] showed that well-fed animals move more slowly in the presence of food than in the absence of food (basal slowing response). Furthermore, when starved animals encounter food, the slowing response is even greater (enhanced slowing response). Both the basal and enhanced slowing responses were normal in the *nmr-1(ak4)* and *nmr-2(ak7)* mutants (data not shown), indicating that these worms can normally sense starvation.

### NMDA-Gated Currents Are Dependent on Both NMR-1 and NMR-2

To test the hypothesis that functional NMDARs in *C. elegans* require both NMR-1 and NMR-2, we measured glutamate- and NMDA-gated currents in AVA interneurons of wild-type worms and *nmr-2* mutants. In wild-type worms, glutamate elicited a rapidly activating inward or outward current that quickly desensitized when the membrane potential was held at either –60 mV or +40 mV, respectively (Figure 4A). This rapid current component is mediated by GLR-1 and GLR-2 AMPARs [29]. A smaller, more slowly desensitizing current component that is known to be dependent on NMR-1 [3] was also observed. The slower outwardly rectifying current was isolated with the specific agonist NMDA (Figure 4B). In *nmr-2(ak7)* worms (Figure 4C), glutamate elicited a rapidly activating and inactivating current, which was similar to glutamate-gated currents recorded from *nmr-1* mutants [3]; however, NMDA-gated currents were not observed (Figure 4D). These data further support the notion that NMR-1 and NMR-2 form a heteromeric NMDAR.

### NMDARs Function in the RIM Interneurons to Facilitate Memory Retention

We next determined in which neurons NMDARs function to facilitate the retention of avoidance memory. *nmr-1* and *nmr-2* are coexpressed in a limited number of neurons [32], including the RIM interneurons, the AVG pioneer neuron, and the command interneurons AVA, AVD, AVE, and PVC that form part of the neural circuit that regulates both forward and backward movement [33]. We expressed wild-type *nmr-1* in a subset of these neurons in transgenic *nmr-1(ak4)* mutants using cell-specific promoters and tested these worms in the salt-chemotaxis-learning assay. The behavior was restored in transgenic *nmr-1* mutants that expressed NMR-1 under the regulation of the *glr-1* promoter that drives expression in all cells that normally express NMDARs (Figure 5A). However, expressing NMR-1 in AVA (*rig-3* promoter), AVD (*tol-1* promoter), or AVG (*odr-2* promoter) did not rescue the memory defect of *nmr-1(ak4)* worms (Figure 5A). Interestingly, avoidance behavior in transgenic mutants that expressed NMR-1 in the RIM interneurons using the *tdc-1* promoter was not significantly different from the behavior observed in wild-type worms (Figure 5A).

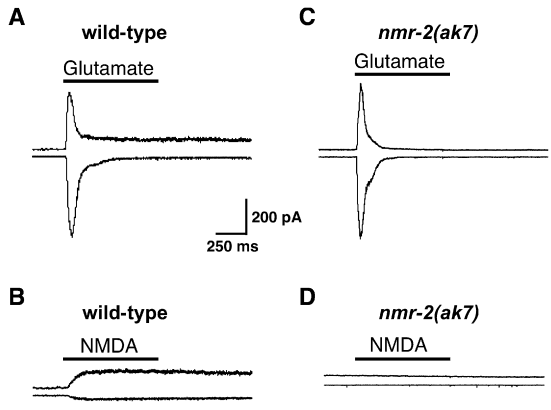


Figure 4. NMR-2 Is Required for NMDA-Gated Currents in the AVA Interneuron

Currents in response to 1 mM glutamate (A and C) or 1 mM NMDA (B and D), recorded from the AVA interneuron held at either –60 or +40 mV in either wild-type (A and B) or *nmr-2(ak7)* (C and D) worms.

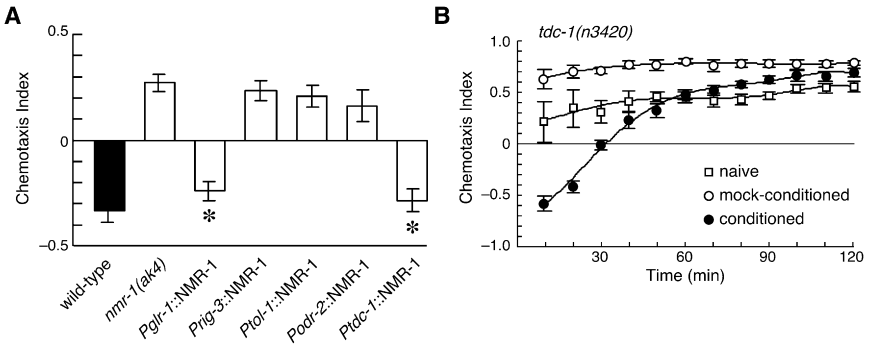


Figure 5. NMDARs Are Required in the RIM Interneurons to Facilitate Memory Retention

(A) Chemotaxis index 40 min after conditioning in wild-type worms (black bar) and in *nmr-1(ak4)* mutants and transgenic *nmr-1* mutants (white bars) that expressed wild-type NMR-1 under the regulation of various cell-specific promoters (n = 4–5). The asterisk denotes a significant difference from *nmr-1(ak4)*, $p < 0.001$.

(B) Chemotaxis learning in *tdc-1(n3420)* mutants (n = 6). *tdc-1* mutants were statistically different from the wild-type at 40 min ($p < 0.005$). Error bars represent SEM.

Together, these data suggest that NMDARs expressed in the RIM interneurons play a crucial role in memory retention in the salt-chemotaxis-learning paradigm.

To determine whether RIM synaptic activity is important for memory retention, we assessed salt chemotaxis learning in *tdc-1(n3420)* mutants. *tdc-1* encodes a tyrosine decarboxylase that is expressed in RIM and necessary for both tyramine and octopamine biosynthesis and neurotransmission [34]. Interestingly, *tdc-1* mutants showed the same memory-retention defects as NMDAR mutants (Figure 5B), suggesting that signaling downstream of RIM occurs via neurotransmission rather than electrical coupling through gap junctions.

## NMR-1 and NMR-2 Are Essential for Memory Retention of a Learned Avoidance Behavior

Using *C. elegans*, we have taken a genetic approach to identifying the cellular and molecular requirements for an associative-learning behavior. Interestingly, we showed that the NMDAR subunits NMR-1 and NMR-2, but not the GLR-1 and GLR-2 AMPAR subunits, are required for the memory of a learned avoidance response. Thus, in salt chemotaxis learning [4], *nmr-1* and *nmr-2* single mutants learned to avoid NaCl after starvation conditioning; however, their memory of this association was impaired, and chemotaxis toward NaCl recovered more rapidly than in wild-type animals. This finding is the first evidence that NMDARs are required for memory retention in *C. elegans*, and it provides insight into the genes and neural circuits that regulate a fundamental process that is conserved across species.

The NMR-1 and NMR-2 subunits are coexpressed in the same subset of neurons and are predicted to form a functional heteromeric NMDA-type iGluR [32]. In support of this hypothesis, we showed that memory defects of the *nmr-1; nmr-2* double mutant were identical to both single mutants, and that like *nmr-1* [3], *nmr-2* is required for NMDA-gated currents in the AVA interneurons. NMR-1 and NMR-2 are expressed in five pairs of interneurons [3, 32] and are required in only one of these, the RIMs, for memory retention of salt avoidance. The RIM interneurons receive input from the ASE salt-sensing neurons via the AIY interneurons. Ablating either AIY or RIM changes the behavior of worms in starved conditions. Wild-type worms transferred to a food-free environment initially execute a high frequency of direction changes (reversals), which gradually diminishes over time [35]. In contrast, worms lacking either AIY or RIM maintain a high reversal frequency under starved conditions [36]. Furthermore, the modifying of reversal behavior has been implicated in navigation processes during taxis behaviors [37–39]. Thus, NMDARs in the RIM interneurons may maintain the association between NaCl and starvation by experience-dependent modification of the reversal frequency. We also showed that *tdc-1* mutants have the same memory defects as *nmr-1* and *nmr-2* mutants, suggesting that the signaling pathway downstream of RIM involves either tyramine or octopamine neurotransmission. Interestingly, octopamine has been shown to modulate associative learning in insects [40–43], and our results suggest that similar mechanisms may exist in *C. elegans*.

NMDARs are thought to facilitate associated learning and memory by acting as coincidence detectors [44]. Thus, activation of vertebrate NMDARs requires two events to happen simultaneously: (1) ligand-binding to the receptor, which causes channel opening, and (2) postsynaptic cell depolarization, which relieves a voltage-dependent $Mg^{2+}$ block of the channel. NMDA-gated currents in *C. elegans* are outwardly rectifying, consistent with a voltage-dependent $Mg^{2+}$ block on the receptor [3]. Interestingly, although GLR-1 and GLR-2 are expressed in the same neurons as NMR-1 and NMR-2, GLR-1 and GLR-2 do not appear to have a central role in salt chemotaxis learning and memory. This suggests that other non-NMDA-type iGluR subunits, e.g., GLR-4 or GLR-5, which are coexpressed with NMR-1 and NMR-2 [32]), may have critical roles in these processes. Contrary to salt chemotaxis learning, GLR-1 is necessary for long-term habituation to vibration stimuli, but a role for NMDARs in this form of learning has not been described [17]. Our findings suggest that two independent signaling pathways regulate the memory of the learning processes of habituation and associative learning. Further genetic analyses of salt chemotaxis learning, including the identification of interacting molecules acting upstream or downstream in the pathway, will help elucidate the neuronal mechanisms of learning and memory acquisition in *C. elegans* and may lead to a better understanding of these important behaviors in more complex organisms.

## Experimental Procedures

### General Methods and Strains

Animals were grown at 20°C unless otherwise noted. All strains were derivatives of the Bristol strain N2 (wild-type). The mutants used in this study were *glr-1(n2461)*, *glr-2(ak10)*, *nmr-1(ak4)*, *nmr-2(ak7)*, and *tdc-1(n3420)*. Transgenic strains were generated by microinjection to achieve germline transformation as previously described [45]. The *nmr-2(ak7)* deletion mutation was generated by imprecise excision of the Tc1 transposon from the *nmr-2* locus. We used PCR to identify Tc1 insertion and subsequent excision. Electrophysiological recordings in vivo from the interneuron AVA were made as previously described [3, 46]. The paired glutamate- and NMDA-gated currents for wild-type and *nmr-2(ak7)* were recorded from the same AVA neuron.

### Salt-Chemotaxis-Learning Assay

Details of the learning assay have been previously described [4]. The animals were washed with 10 mM MOPS buffer, placed on a conditioning plate

(10 mM MOPS-$NH_4$ [pH 7.2], 50 mM NaCl, and 3% agar) or a mock-conditioned plate (10 mM MOPS-$NH_4$ [pH 7.2] and 3% agar), and incubated at 20°C for 4 hr. The animals were again collected, and we assayed chemotaxis by placing them at the center of a 6 cm plate on chemotaxis agar (10 mM MOPS-$NH_4$ [pH 7.2] and 3% agar), on which a salt gradient had been formed for 19–23 hr by placing an agar plug containing 50 mM NaCl at one end of the plate. Thereafter, the number of animals was counted every 10 min for a total of 4 hr. The chemotaxis index was calculated as previously described [13], (A − B) / (A + B), where A was the number of animals on the NaCl side of the plate and B was the number of animals on the opposite side (Figure 1A). To account for worms that died or that were not able to move, we did not count animals that remained at the starting point. Student's t test or ANOVA was used to determine statistical significance. Error bars throughout represent the SEM.

### Plasmids

The various promoter fusions to *nmr-1* coding sequences were constructed with the GATEWAY system (Invitrogen). To construct entry vectors carrying a promoter sequence, we amplified the promoter regions by PCR from *C. elegans* genomic DNA and then inserted them into the pDONR201 vector by site-specific recombination. Promoter fragments were 5.3 kb *glr-1*, 4 kb *rig-3*, 5.5 kb *tol-1*, 5 kb *odr-2*, and 4.5 kb *tdc-1*. To generate destination vectors, we amplified *nmr-1* coding sequences from first-strand cDNA and subcloned them into the KpnI sites of the pPDDEST vector. The oligonucleotides used to amplify *nmr-1* were 5′-CAGATATGTTCCGAATATCAGTTA-3′ (sense) and 5′-CACATAAAATCTAGTTGATCTTGCT-3′ (antisense). The cosmid T01C3 contains an open reading frame predicted to encode an NMDAR subunit (NMR-2). We identified the authentic 5′ end of *nmr-2* by PCR amplification from first-strand *C. elegans* cDNA with spice leader SL1-specific oligonucleotides. Analysis of the predicted NMR-2 protein sequence was done with the ExPASy Proteomics suite of programs [47].

### Accession Numbers

The GenBank accession number for the *nmr-2* cDNA sequence reported in this paper is AF318614.

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